Phospholipase C–Mediated Signaling Is Altered During HaCaT Cell Proliferation and Differentiation

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To elucidate the signaling mechanisms associated with keratinocyte differentiation, we studied *in vitro* phospholipase C–mediated signal transduction, which results in the generation of inositol phosphates, comparing proliferating *versus* differentiated HaCaT cells, a human keratinocyte line. Bradykinin- or A23187-induced formation of inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate, and inositol monophosphates, as determined by anion exchange high performance liquid chromatography, were found to be highest in the early logarithmic growth phase of the cells. In more highly differentiated HaCaT cells, which expressed maximal amounts of the differentiation marker involucrin, inositol phosphate formation was reduced to about one third of that in proliferating cells. Thin layer chromatography of membrane phosphatidylinositol phosphates revealed that this reduction was associated with a steady decrease in phospholipase C substrates. Immunoblot analysis of phospholipase C isozymes, however, and of expression of Gqα, the G protein subunit that activates phospholipase Cβ, revealed no decrease during the differentiation phase. The results suggest that the inositol-phospholipid signal transduction pathway is involved in keratinocyte proliferation and in the induction of differentiation, with attenuated signal transduction activity via phospholipase C–coupled receptors in more differentiated keratinocytes. ***Key words: keratinocytes/inositol phosphates/inositol phospholipids. J Invest Dermatol 108:748–752, 1997***

Several receptors are known to transduce their specific signals via phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol bisphosphate, resulting in the generation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (DAG). $Ins(1,4,5)P_3$ releases intracellular free Ca^{2+} from its receptor sites, and DAG is the endogenous activator of several protein kinase C isozymes (Berridge, 1987b; Nishizuka, 1988; Meldrum *et al*, 1991). Based primarily on the observation that several receptors for growth factors and mitogens use this signal transduction pathway, it was assumed that PLC activation plays a major role in the regulation of cellular proliferation (Berridge, 1987a, 1987b; Michell, 1992). Further support for this hypothesis was provided by other studies, e.g., with quail embryo cell cultures, in which the breakdown of phosphatidylinositol was slowed when cell growth was restricted (Diringer and Friis, 1977), or with NIH 3T3 fibroblasts, in which microinjection of $PLC\gamma_1$ initiated DNA replication that was inhibited by coinjection of antibodies to $PLC\gamma_1$ (Smith *et al*, 1990).

More recent observations also suggest a possible role of the PLC-dependent signal transduction pathway in the regulation of cell differentiation. Studies performed in retinoic acid–induced granulocytic differentiation of promyelocytic HL-60 cells revealed early inhibition of the PLC-mediated release of $Ins(1,4,5)P_3$ and DAG (Geny *et al*, 1991; Porfiri *et al*, 1991). With an onset of 10 min, this phenomenon seems to precede all other events associated with the differentiation of HL-60 cells toward neutrophils (Geny *et al*, 1991). A similar observation was made in studies of retinoic acid–induced differentiation of neuroblastoma cell lines, in which a rapid decrease in $Ins(1,4,5)P_3$ and DAG levels occurred within 1 min after treatment with 1 μM retinoic acid (Ponzoni *et al*, 1991).

In the current study, we investigated whether a similar down-regulation of inositol lipid metabolites occurs during the natural course of proliferation and differentiation in human keratinocytes, in the absence of any external pharmacologic modulation. HaCaT cells represent a spontaneously immortalized cell line, and they closely approximate normal human keratinocytes in their capacity to differentiate in culture (Boukamp *et al*, 1988; Breitkreutz *et al*, 1993). Because this cell line allows a more reproducible and consistent study of changes during the process of early differentiation than do freshly isolated keratinocytes from different donors and body regions, we have used this keratinocyte line to study changes in PLC-mediated signal transduction. HaCaT cells have been shown previously to possess a functionally active PLC signaling system (Rosenbach *et al*, 1993; Haase *et al*, 1996) with properties similar to those described in human SCC-12F keratinocytes (Rosenbach and Greenlee, 1991) and in human primary keratinocyte cultures (Talwar *et al*, 1989, 1990).

MATERIALS AND METHODS

Cell Culture Cell culture was performed as described elsewhere (Wanner *et al*, 1995). Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, Deutsches Krebs Forschungszentrum,, Heidelberg, Germany) were seeded at a density of 2×10^4 cells/cm^2 in either 35-mm or 150-mm cell culture dishes (Falcon, Darmstadt, Germany) and were maintained in 2 ml Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein,

Manuscript received December 4, 1996; revised January 6, 1997; accepted for publication January 29, 1997.

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Abbreviations: $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $Ins(1,4)P_2$, inositol 1,4-bisphosphate.

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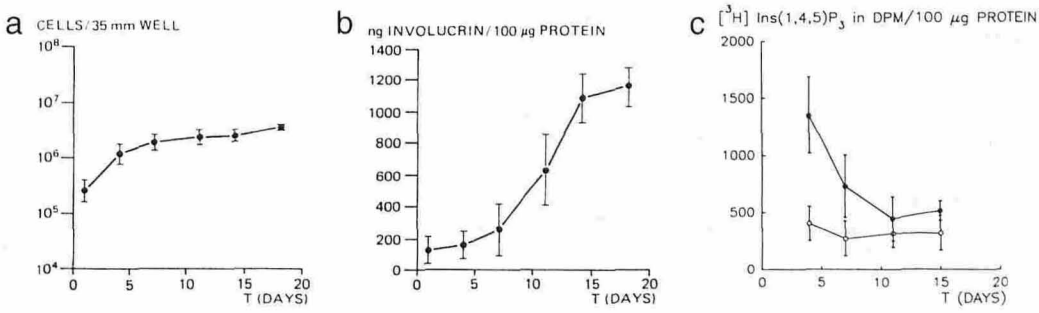

Figure 1. Reduced Ins(1,4,5)P_3 formation in HaCaT cells proceeding from proliferation to differentiation. Time course (days of culture) of HaCaT cell proliferation *(a)*, their differentiation measured by an involucrin enzyme-linked immunosorbent assay *(b)*, and their Ins(1,4,5)P_3 formation *(c)*. ●, cells stimulated with 10 µM bradykinin; ○, vehicle controls *(c)*. Results represent data from five independent experiments, performed in duplicate and expressed as mean ± SD.

Germany) per 35-mm well, supplemented with 5% fetal bovine serum (Life Technologies) and 4 mM glutamine (Life Technologies). Feeding was performed every 3–4 d. Cell growth was determined by cell counts in a hemocytometer.

Protein and Involucrin Assays Total protein amount was quantified using the Pierce (Pierce, Munich, Germany) protein assay. Measurement of involucrin, a marker protein for keratinocyte differentiation, was performed using an enzyme-linked immunosorbent assay (Paesel, Frankfurt, Germany) according to the manufacturer's instructions.

Measurements of Inositol Phosphates Cells were labeled by adding 40 µCi of myo-[2-^{3}H]inositol (19 Ci/mmol) (Amersham, Braunschweig, Germany) per 35-mm well to the cell culture medium 48 h before the experiment. Alternatively, 40 µCi myo-[2-^{3}H]inositol per 35-mm well was added when the cells were seeded and was replaced with every change of the cell culture medium. Before the experiment, the cells were washed twice with phosphate-buffered saline, followed by a pre-incubation at 37°C for 30 min in Earle's balanced salt solution supplemented with 10 mM LiCl, a known inhibitor of inositol monophosphatase (Majerus *et al*, 1988), and 20 mM HEPES (Sigma, Deisenhofen, Germany), pH 7.4. Incubations were started at 37°C by the addition of test substances dissolved in the same buffer and were stopped by aspiration of the buffer and addition of 300 µl ice-cold 10% $HClO_4$ (Merck, Darmstadt, Germany). The cells were scraped from the dishes and disrupted by three cycles of freezing and thawing. After centrifugation at 12,000 × *g* for 6 min, neutralization of the supernatants was performed with 10 M KOH or 1 M KOH (Merck). The sample was again centrifuged at 12,000 × *g* for 6 min, and the supernatant was stored at −20°C until analysis. Separation of inositol phosphates was achieved with a Partisphere SAX column (Whatman, Wiesloch, Germany) using an ammonium dihydrogen phosphate gradient, as described previously (Rosenbach and Greenlee, 1991). Radioactivity was monitored on-line using a Ramona LS detector (Raytest, Munich, Germany) and Flo-Scint IV as scintillant (Packard, Frankfurt, Germany). Integrated peak areas were calculated in dpm and were related to the protein content of the sample. Identification of inositol phosphates was performed by comparison with authentic tritiated standards (Amersham).

Analysis of Phosphatidylinositol Phosphates Cells were labeled with 10 µCi myo-[2-^{3}H]inositol per 35-mm well and incubated as described previously. The dishes were washed twice with phosphate-buffered saline followed by the addition of 900 µl water:methanol:HCl (100:100:1; v/v/v) (Merck). After scraping the cells off the surface and transferring them into test tubes, we extracted phospholipids by the addition of 900 µl chloroform (Merck) and then incubated the samples for 30 min in a rocker at room temperature. After a centrifugation step at 8000 × *g* for 10 min, the lower phase was collected and dried under a stream of nitrogen. Dried extract was dissolved in 20 µl chloroform:methanol:water (75:25:2; vol/vol/vol) (Merck) and transferred to potassium oxalate–impregnated high performance thin layer chromatography plates (Merck). Chloroform:acetone: methanol:acetic acid:water (Merck) (120:45:39:31:21; v/v/v/v/v) served as solvent system. Phosphatidylinositol phosphates were visualized with iodine vapor and identified by comparison with tritiated (Amersham) and nontritiated standards (Boehringer). Radioactivity was quantified by scraping the plate areas and subsequent liquid scintillation counting.

Immunoblot Analysis Keratinocytes were washed twice with phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) containing 1 mM phenylmethylsulfonyl fluoride (Merck) and were then scraped from the culture dish. Total cell homogenate was prepared by three strokes of an ultrasound homogenizer (Branson, Danbury, CT) for 30 s. The sample was solubilized in sodium dodecyl sulfate sample buffer, yielding a final concentration of 2% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 10 mM Tris (Sigma), pH 6.8. Aliquots containing equal amounts of protein were heated at 95°C for 5 min and applied to 10% or 8% polyacrylamide gels. The separated proteins were transferred to polyvinyldifluoride membranes (Du Pont) as described previously (Möller *et al*, 1993). For identification of immunoreactivity, we incubated the membranes for 1 h with polyclonal antibodies directed against the various proteins. Immunostaining was performed by incubation with horseradish peroxidase–coupled goat anti-rabbit IgG (Dianova, Hamburg, Germany) at a dilution of 1:2000 and subsequent visualization on x-ray films (Kodak) using the Renaissance chemoluminescence detection system (Du Pont, Bad Homburg, Germany). Dilutions of the primary antibodies were as follows: anti-Gqα, 500; anti-PLCβ_3, 1:500; and anti-PLCγ_1, 1:500. The antibody against Gqα was a kind gift from Dr. John Exton (Nashville, TN). The antibodies against PLC isozymes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Inositol Phosphate Formation Is Reduced in More Differentiated HaCaT Keratinocytes HaCaT keratinocyte proliferation approximated logarithmic growth during the initial culture phase. The cells reached confluence on day 6, and cell numbers remained at a plateau thereafter with only a minor increase **(Fig 1*a*)**. Determination of keratinocyte differentiation by involucrin measurements revealed a linear increase in this marker protein beginning on day 7 (end of the proliferative phase) of cell culture and lasting until day 14 **(Fig 1*b*)**. After this time, involucrin expression remained at the same level until day 18 of cell culture, which was the latest time point examined.

Time course studies of inositol phosphate formation in both proliferating and more differentiated HaCaT keratinocytes upon stimulation with bradykinin or calcium ionophore A23187 revealed maximal levels for Ins(1,4,5)P_3 generation at 20 s, and for its dephosphorylation products inositol 1,4-bisphosphate (Ins(1,4)P_2) and inositol monophosphate at 1 min and 30 min, respectively (data not shown). These time points were used for studying product formation for each metabolite in all further experiments. Ins(1,4,5)P_3 formation induced by 10 µM bradykinin was highest at the beginning of the proliferative phase of HaCaT keratinocytes at day 4, the earliest time examined **(Fig 1*c*)**. Formation of Ins(1,4,5)P_3 decreased steadily from approximately 1500 dpm per 100 µg protein to approximately 500 dpm at day 11 of cell culture and remained at this level until day 15 **(Fig 1*c*)**.

Bradykinin-induced Ins(1,4,5)P_3, Ins(1,4)P_2, and inositol monophosphate formation in proliferating keratinocytes (day 4 of cell culture) was compared with that in differentiated keratinocytes (day 15 of cell culture). Maximal formation of each metabolite was determined to be reduced to a range of 20–30% in differentiated keratinocytes as compared with proliferating keratinocytes **(Fig**

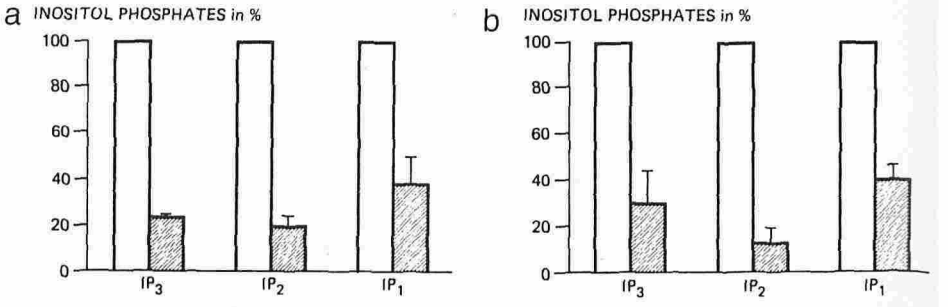

Figure 2. Attenuation of inositol phosphate generation is bradykinin receptor-independent. Inositol phosphate formation was induced with 10 μM bradykinin *(a)* or 10 μM A23187 *(b)*. Ins(1,4,5)P_3 (IP_3) was measured at 20 s, Ins(1,4)P_2 (IP_2) at 2 min, and inositol monophosphate (IP_1) at 30 min in proliferating (day 4, □) *versus* differentiated keratinocytes (day 15, ▨). Results were measured in dpm/10^6 cells and are expressed as percentage of control. Data represent the mean ± SD of three independent experiments, performed in duplicate.

2*a*). HaCaT cells were furthermore stimulated by direct activation of PLC with 10 μM calcium ionophore A23187. Again, a decrease of each inositol phosphate metabolite was observed in differentiated HaCaT cells **(Fig 2*b*)**.

Expression of Gqα and PLC Isozymes Is Not Reduced During Early Differentiation The possible involvement of Gqα, the G protein subunit that activates phosphoinositide-specific PLC, was analyzed in immunoblot studies. Proliferating keratinocytes exhibited a band at the 42-kDa position, the reported molecular mass of this G protein subunit (Smrcka *et al*, 1991). Expression of Gqα was even slightly enhanced in more differentiated HaCaT cells at day 15 of cell culture **(Fig 3)**.

Western blot studies with affinity-purified, polyclonal rabbit antibodies against PLC isozymes are shown in **Fig 4**. PLCβ_3 and PLCγ_1 revealed strong signals at the reported molecular weights (about 150 kDa for PLCβ_3 and 148.5 kDa for PLCγ_1). Analysis of the β_3 and γ_1 isoform expression during HaCaT cell proliferation and differentiation showed no significant changes in the detectable amounts of these isozymes **(Fig 4)**. No specific signals were obtained by incubation of blotted HaCaT cell lysates with antibodies against PLCβ_1, PLCβ_2, PLCβ_4, PLCγ_2, or the δ isoforms of PLC. In contrast, clear bands at the corresponding molecular weights of these isozymes, except for PLCβ_4, were detectable in commercially available tissue lysates of human brain, heart, lung, liver, kidney, and placenta (ITC Biotechnology, Heidelberg, Germany), which served as positive controls (data not shown). The PLCβ_4 isozyme so far has only been described in retinal tissue (Lee *et al*, 1994; Jiang *et al*, 1994) and is thus not likely to play an important role in inositol lipid-mediated signaling in keratinocytes.

The Amount of Phosphatidylinositol Phosphates Is Decreased in More Differentiated Keratinocytes Analysis of the total amount of membrane phosphatidylinositol phosphates exhibited a steady decrease of these PLC substrates during proliferation and differentiation **(Fig 5)**. Maximal values were obtained in proliferating keratinocytes at day 4. The corresponding values for more differentiated keratinocytes (day 15) declined to 27% for phosphatidylinositol and to 41% and 39% for phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate, respectively. Incubation of the cells with myo-[2-^{3}H]-inositol from the day of seeding yielded similar results (data not shown).

DISCUSSION

In the current investigation, we report a reduction of bradykinin- and A23187-induced inositol phosphate formation during HaCaT cell proliferation and differentiation. Although proliferating HaCaT cells show a pronounced generation of Ins(1,4,5)P_3 upon stimulation with bradykinin or A23187, this response is subsequently diminished with the ongoing process of differentiation.

Such a phenomenon has not been described before in human keratinocytes or HaCaT cells and contrasts with results obtained in primary murine keratinocytes (Jaken and Yuspa, 1988; Tang *et al*, 1988; Lee and Yuspa, 1991a, 1991b). In murine cells, calcium-

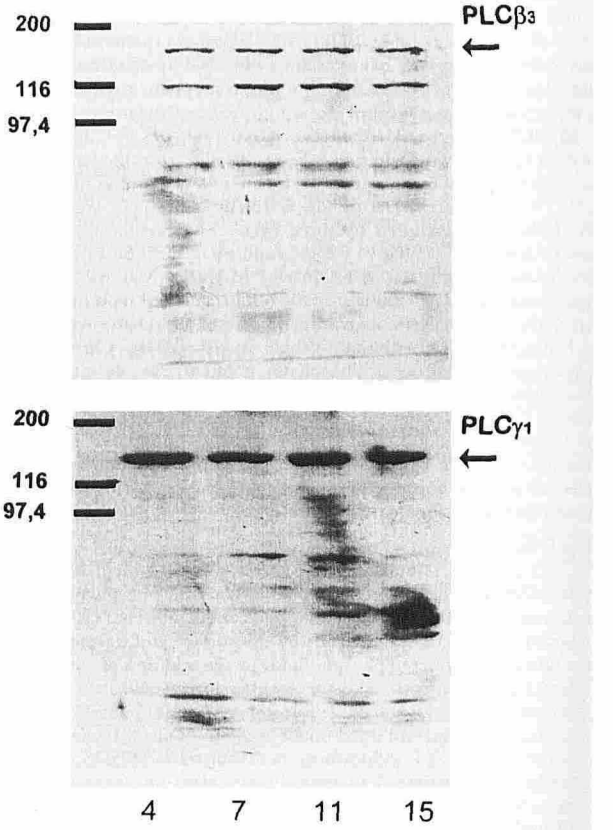

Figure 4. Unchanged expression of PLC isozymes in proliferating and differentiating HaCaT cells. Immunoblot analysis of PLC isozymes in HaCaT keratinocytes. The numbers below the lanes indicate the day of cell culture. Day 4 represents proliferating, day 7 confluent, and days 11 and 15 more differentiated cells. Data show representative results of two experiments.

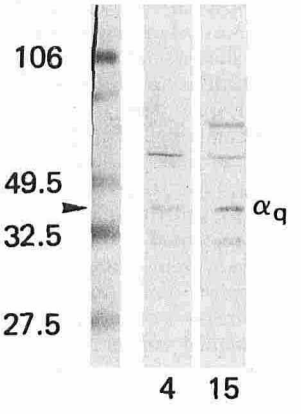

Figure 3. Similar expression of Gqα in proliferating and differentiating HaCaT cells. Immunoblot analysis of Gqα expression in proliferating keratinocytes (day 4 of cell culture) *versus* differentiated keratinocytes (day 15 of culture). Whole-cell homogenates at 100 μg were loaded onto a sodium dodecyl sulfate-polyacrylamide gel, and proteins were transferred to nitrocellulose. *Left lane* represents the position of molecular-weight standards. ➤ indicates 42 kDa, the reported molecular weight of Gqα. Data show representative results from three separate experiments.

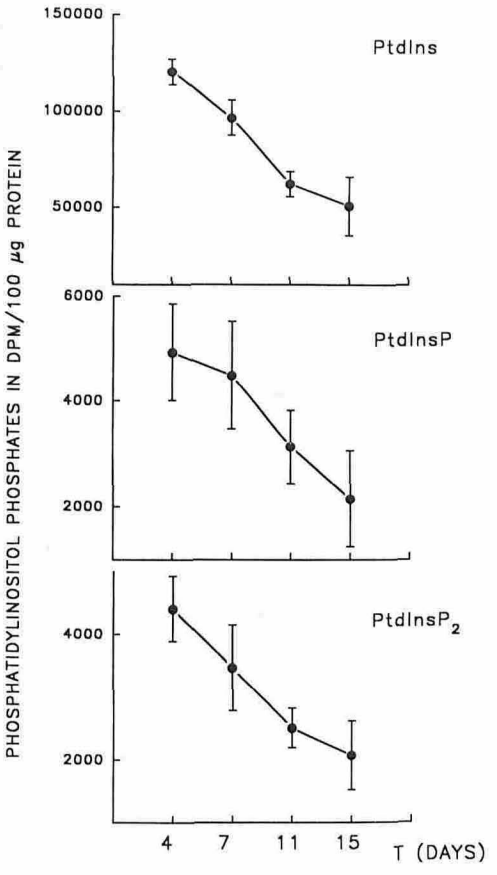

Figure 5. Reduced levels of phosphatidylinositol phosphates in differentiating *versus* proliferating HaCaT keratinocytes. Analysis of myo-[2-^{3}H]-inositol–labeled keratinocytes for phosphatidylinositol (PtdIns), phosphatidylinositol phosphate (PtdInsP), and phosphatidylinositol bisphosphate ($PtdInsP_2$) from different days of cell culture by thin layer chromatography. The *x axis* indicates the day of cell culture. Results are shown in dpm/100 µg protein (mean ± SD; n = 3).

induced differentiation was associated primarily with an increase in inositol phosphate production. A link between enhanced PLC-mediated signal transduction and the process of calcium-induced differentiation has not been proved, however. Data from neoplastic murine keratinocytes revealed that an increase in the extracellular calcium concentration does not induce differentiation in these keratinocytes, but results nevertheless in a marked enhancement of inositol phosphate generation (Lee and Yuspa, 1991a). The latter finding corroborates results obtained in human keratinocytes (Pillai and Bikle, 1992). These authors found enhanced $Ins(1,4,5)P_3$ generation upon incubation with both adenosine triphosphate and increased extracellular calcium. Only increasing the extracellular calcium concentration induced differentiation in this model, however, and incubation with adenosine triphosphate was not effective. One must further consider that all of the data cited were obtained from calcium-induced keratinocyte differentiation. This is in contrast to our model, in which HaCaT cells are able to differentiate spontaneously under constant culture conditions without abrupt changes of the extracellular calcium concentration. An elevation of extracellular Ca^{2+} should interfere with the intracellular calcium homeostasis and could thus enhance PLC activity, as this enzyme is Ca^{2+}-regulated (Rhee and Choi, 1992). Species differences between murine and human keratinocytes in the regulation of keratinocyte differentiation or inositol lipid signaling might further contribute to the described divergences.

To elucidate the mechanisms involved in the decreased generation of $Ins(1,4,5)P_3$ in more differentiated HaCaT keratinocytes, we investigated the different components of the inositol lipid signaling system in these cells. The observed results could have been due to a downregulation of the bradykinin receptor. The Ca^{2+}-mediated activation of PLC was therefore examined by means of A23187 stimulation of HaCaT keratinocytes and yielded similar results, suggesting a receptor-independent mechanism.

A further possibility could have been a reduced expression of G proteins. G proteins are heterotrimeric and composed of α, β, and γ subunits. They transduce signals between seven–transmembrane segment receptors and intracellular effectors, such as adenyl cyclases, ion channels, and phospholipases (Gilman, 1987; Birnbaumer *et al*, 1990; Simon *et al*, 1991). The γ-type PLC isozymes are activated by receptor-coupled tyrosine kinases without involvement of G proteins (Rhee and Choi, 1992). In contrast, α subunits of the Gq class of G proteins have been identified to activate specifically β-type PLC isozymes (Smrcka *et al*, 1991; Taylor *et al*, 1991; Waldo *et al*, 1991; Jhon *et al*, 1993), which transduce the signal of seven–transmembrane domain receptors such as the one for bradykinin (Speziale *et al*, 1985). The bradykinin-induced release of $Ins(1,4,5)P_3$ was not sensitive to adenosine diphosphate ribosylation by pertussis toxin (data not shown), indicating that a pertussis toxin–insensitive G protein of the Gq class is involved in the regulation of PLCβ in HaCaT cells. Changes in the expression of α subunits due to the proliferative or differentiated state of the cell have been described in adipocytes and F9 teratocarcinoma cells for the Gs and Gi classes, which are coupled to adenyl cyclases (Watson *et al*, 1992; Kilgour and Anderson, 1993). In contrast to these observations with G_s and G_i isoforms, knowledge about proliferation- and differentiation-dependent alterations in the expression of α subunits of the Gq class are still scarce. In the current study, we found a slight enhancement in Gqα expression in more differentiated HaCaT cells, making it unlikely that Gqα contributes to diminished inositol phosphate formation.

Nine PLC isozymes have been isolated from mammalian tissues. The two SH domain–containing γ isoforms are regulated via tyrosine phosphorylation, whereas the β isoforms are activated by G proteins. The function of PLCδ is still not clear (Rhee and Choi, 1992). HaCaT cells express only PLCβ_3 and PLCγ_1, as determined by Western blot studies, with no significant differences in proliferating *versus* differentiating cells. Reduced $Ins(1,4,5)P_3$ release thus is apparently not caused by a decrease in PLC expression. Furthermore, alterations in PLC expression have been found in only a few cellular systems, with contradictory results. A correlation between elevated PLCγ_1 expression and an increased degree of malignancy was described in colorectal epithelial neoplasia (Noh *et al*, 1994; Park *et al*, 1994), whereas a decrease in PLCβ both at the protein and mRNA level, but not in PLCγ, was detected in the nuclei of Friend erythroleukemia cells induced to differentiate by dimethylsulfoxide (Martelli *et al*, 1994). In cultured murine keratinocytes, calcium-induced differentiation led to a 2- to 3-fold increase in PLCγ_1 and PLCδ_1 protein (Punnonen *et al*, 1993). The calcium shift in the medium (from 0.05 mM to 1.4 mM) might, however, interfere with the calcium homeostasis of these cells, and this model thus cannot be compared directly with the cell culture model used in the present study.

Our data show that reduced levels of inositol phosphates **(Fig 1*c*)** are associated with similar changes of the substrate of PLC, the membrane phospholipid phosphatidylinositol bisphosphate, and its precursors phosphatidylinositol phosphate and phosphatidylinositol **(Fig 5)**. To rule out that these phenomena are due to a reduced incorporation of myo-inositol in more differentiated keratinocytes, experiments were performed by adding myo-[2-^{3}H]-inositol when the keratinocytes were seeded, replacing it with every change of the cell culture medium. This set of experiments yielded the same results as the ones with a 48-h labeling period. These data are in agreement with both *in vitro* and *in vivo* findings showing a gradual decrease in the rate of phospholipid synthesis and in phospholipid content during keratinocyte differentiation (Ponec, 1994). A relation between phosphatidylinositol metabolism and cell growth and differentiation is further supported by data obtained in Japanese

quail embryo cell cultures, in which cell growth correlated with the breakdown of ^{32}P-labeled phosphatidylinositol (Diringer and Friis, 1977). Likewise, breakdown of phosphatidylinositol was decreased in chicken lens epithelial cells after the induction of differentiation (Vu *et al*, 1983).

Our results provide reasons to assume that a decrease in PLC-mediated signaling might also play a role in the regulation of human keratinocyte differentiation. A function of reduced inositol phosphate formation in the induction of differentiation is supported by studies in HL-60 cells (Faletto *et al*, 1985; Geny *et al*, 1991; Porfiri *et al*, 1991), which revealed decreased inositol phosphate levels and diminished DAG generation upon retinoic acid– or dimethylsulfoxide-induced differentiation. Studies in another cellular model of retinoic acid–induced differentiation, the human neuroblastoma cell lines LAN-1 and GI-ME-N, led to similar results (Ponzoni *et al*, 1991).

In conclusion, if HaCaT cells reflect changes in primary keratinocytes, our results suggest that inositol phosphate formation is reduced during the course of keratinocyte proliferation and differentiation. The observed effect is due to decreased levels of membrane phosphatidylinositol phosphate. On the basis of similar observations in retinoic acid–induced HL-60 cell and neuroblastoma cell differentiation, we propose a role for diminished inositol phosphate formation in the switch from keratinocyte proliferation to induction of differentiation. This hypothesis needs further experimental proof, however. Our results furthermore suggest attenuated responsiveness of more highly differentiated keratinocytes to external receptor ligands using this signal transduction pathway.

This study was supported in part by Deutsche Forschungsgemeinschaft Grant Ro 695/4–1 and 4–2 and by a graduate fellowship from the Freie Universität Berlin (NaFög) for C. Liesegang. We express our thanks to Dr. N.E. Fusenig (Deutsches Krebs Forschungszentrum, Heidelberg, Germany) for providing the HaCaT keratinocytes, to Dr. J. Exton (Vanderbilt University, Nashville, TN) for providing the antibody against Gqα, and to Dr. G. Kolde (Department of Dermatology, Humboldt Universität Berlin) and Dr. G. Schultz (Department of Pharmacology, Freie Universität Berlin) for helpful comments during the preparation of the manuscript.

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